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PARASITEMIA DUE TO *SARCOCYSTIS NEURONA*-LIKE INFECTION IN A CLINICALLY ILL DOMESTIC CAT

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Abstract

An 8-year-old, 6-kg, male neutered Domestic Shorthair cat was presented to The Ohio State University Veterinary Medical Center (OSU-VMC) for difficulty breathing. Physical examination and thoracic radiographs indicated pneumonia, a soft tissue mass in the left caudal lung lobe, and diffuse pleural effusion. The effusion was classified as modified transudate. Rare extracellular elongated (~5–7µm×1–2µm) zoites with a central round to oval-shaped purple to deep purple vesicular nucleus with coarsely stippled chromatin and light blue cytoplasm were seen on a peripheral blood smear. Serum IgG and IgM were positive for *Sarcocystis* sp. antibodies and negative for *Toxoplasma gondii* antibodies, suggesting the infection was acute rather than a recrudescence of prior infection. This organism was most consistent with either *Sarcocystis neurona* or *Sarcocystis dasyi* based on DNA sequence analysis of PCR products using COC ssRNA, ITS-1, snSAG2, and JNB25/JD396 primer sets. This is the first report to visualize by light microscopy circulating *Sarcocystis* sp. merozoites in the peripheral blood of a domestic cat. Therefore, *Sarcocystis* should be considered as a differential diagnosis in cats with suspected systemic protozoal infection.

Keywords

Sarcocystis; feline; peripheral blood; molecular analysis; protozoa

CASE PRESENTATION

An 8-year-old, 6-kg, male neutered Domestic Shorthair cat was presented to the referring veterinarian for lethargy and decreased appetite of several days duration. The indoor-outdoor

cat had a history of fighting with other stray cats. The cat was febrile (temperature unknown) and had a moderate leukocytosis ($23.1 \times 10^9/L$, RI 3.5–16.0 $\times 10^9/L$) due to moderate neutrophilia ($20.0 \times 10^9/L$, RI 2.5–8.5 $\times 10^9/L$), moderate thrombocytopenia ($58.0 \times 10^9/L$, RI 200–500 $\times 10^9/L$), mildly elevated AST (184 IU/L, RI 10–100 IU/L) and ALT (204 IU/L, RI= 10–100 IU/L) activities, and mild hyperbilirubinemia (1.9 mg/dL, RI=0.1–0.4 mg/dL). The cat was treated with systemic antibiotics (Convenia, dose unknown, Zoetis, Inc., Florham Park, NJ, USA) and was taken home.

The cat was presented to The Ohio State University Veterinary Medical Center (OSU-VMC) several days later for difficulty breathing. The cat was dull to obtunded, 5% dehydrated, normothermic, tachypneic, and dyspneic. Physical examination revealed muffled lung sounds in all ventral lung fields and hepatomegaly. Thoracic radiographs showed an irregular, cavitated soft tissue mass in the left caudal lung lobe, a focal alveolar pattern in the right and left cranial lung lobes, and diffuse pleural effusion. Yellow, hazy fluid was removed from the pleural cavity bilaterally, 150 mL (right side) and 105 mL (left side) initially, followed by an additional 105 mL (right) and 100 mL (left) later that evening. Fluid was classified as a modified transudate (140 cells/ μL , RI <1000 cells/ μL ; 3.6 g/dL total protein, RI <2.5 g/dL). Samples were submitted for a CBC, serum biochemical profile, and coagulation testing. Pertinent CBC and coagulation testing results are listed in Table 1. The CBC was performed using an Advia 2120i hematology analyzer (Siemens Healthcare, Erlangen, Germany). A manual leukocyte differential was performed by a laboratory technologist and confirmed by one of the authors (N.Z.). Coagulation testing was performed on a STACompact (Diagnostica Stago, Parsippany, NJ, USA).

The plasma was moderately icteric, likely due to liver disease. The cat had a moderate thrombocytopenia with macroplatelets and a mildly increased MPV. No platelet clumps were present. The thrombocytopenia and markedly prolonged APTT and PT were indicative of disseminated intravascular coagulation (DIC) or other consumptive coagulopathies. The cat had a moderate leukocytosis characterized by a moderate neutrophilia with a regenerative left shift and evidence of toxic change (Döhle bodies, cytoplasmic basophilia, and cytoplasmic vacuolation), indicative of an inflammatory leukogram. Moderate numbers of reactive lymphocytes and occasional granular lymphocytes were indicative of antigenic stimulation.

Rare extracellular elongated ($\sim 5\text{--}7\mu m \times 1\text{--}2\mu m$) organisms (zoites) with a central round to oval-shaped purple to deep purple vesicular nucleus with coarsely stippled chromatin and light blue cytoplasm were present on the peripheral blood smear (Figure 1A and Figure 1B). The zoites were occasionally found intracellularly within lymphocytes (Figure 1C) and rarely within large mononuclear cells (Figure 1D) and neutrophils. The morphology of the organisms was consistent with *Coccidia* tachyzoites or merozoites. Differential diagnoses included *Toxoplasma gondii*, *Neospora caninum*, and *Sarcocystis* sp, with *Toxoplasma gondii* being the primary differential diagnosis.

Relevant serum biochemistry results are listed in Table 2. Electrolytes, glucose, and lactate were evaluated using the Stat Profile pHox Analyzer (Nova Biomedical, Waltham, MA, USA). All remaining biochemical tests were performed on Cobas 6000 and c501 analyzers

(Roche Diagnostics International Ltd, Rotkreuz, Switzerland). There was a moderate elevation in CK activity, indicative of muscle damage. The mild to moderate elevation in ALT and moderate to marked elevation in AST were likely caused by a combination of muscle and hepatocellular damage. The cat was moderately hyperbilirubinemic with a mild elevation in GGT activity indicative of cholestasis secondary to hepatocellular damage. The panhypoproteinemia was likely due to a combination of protein accumulation in the thoracic effusion, decreased hepatic production, and inflammation given albumin is a negative acute phase protein. The mild hyponatremia with concurrent proportional hypochloremia were also likely due to the thoracic effusion; there was no evidence of renal or gastrointestinal loss. The mildly elevated glucose was indicative of a catecholamine-induced hyperglycemia. The high-normal lactate was likely due to poor tissue perfusion from dehydration and dyspnea. A FeLV/FIV ELISA (IDEXX Laboratories, Westbrook, ME, USA) indicated the cat was FIV antibody positive and FeLV antigen negative. The FIV vaccination history was unknown and no further FIV diagnostic testing was performed. The owners elected humane euthanasia and no necropsy. Additional blood was collected in EDTA tubes for supplemental diagnostics and stored at 4°C. All remaining serum from the biochemistry profile was stored at -20°C until additional diagnostics could be performed.

Given the clinical presentation of the cat and the high seroprevalence of *Toxoplasma gondii* in domestic cats, the primary differential diagnosis was an acute *Toxoplasma gondii* infection.¹ Whole EDTA blood and serum submitted to the Colorado State University Veterinary Diagnostic Laboratory (Fort Collins, CO, USA) for *Toxoplasma gondii* PCR and antibody titer; all of which were negative (PCR negative, IgG and IgM both <1:20).

Immunofluorescence and immunocytochemical staining using *Sarcocystis neurona* monoclonal antibody 2G5-2 was used to further characterize the zoites.² This antibody was previously characterized by A.M. and is not commercially available. Unstained unfixed peripheral blood smears, prepared within 4 h of sample collection and stored at room temperature, were evaluated. Controls included blood smears from a healthy cat and blood spiked with culture-derived *S. neurona*-like SN-MUCAT2 merozoites.^{2,3} For immunofluorescence, slides were fixed in methanol and staining was performed as previously described.^{2,3} Slides were then incubated with mAb 2G5-2 for 30 min at 37°C in a humid chamber. Slides were rinsed with phosphate-buffered saline (PBS) to remove any unbound antibody. Slides were then incubated with fluorescein isothiocyanate (FITC)-conjugated horse anti-mouse IgG (cat #FI-2000, Vector Laboratories, Burlingame, CA, USA) and evaluated on an Olympus BX41 fluorescent microscope (Olympus Global, Shinjuku, Tokyo, Japan). For the immunocytochemistry, a Dako autostainer (Agilent Technologies, Glostrup, Denmark) was used. The slides were fixed in 100% acetone for 10 min. Slides were then incubated with 2G5-2 antibody diluted 1:500 in PBS for 30 min, followed by biotinylated horse anti-mouse IgG (cat #BA-2000, Vector Laboratories, Burlingame, CA, USA) and 3'3'-diaminobenzidine horseradish peroxidase (DAB-HRP) for detection. The *S. neurona* monoclonal 2G5-2 stained the parasites on the blood smear, including what appeared to be intracellular forms not readily apparent by the Wright-Giemsa stain. This could be seen on both the immunofluorescence (Figure 2A) and immunocytochemical (Figure 2B) staining.

Antibody response to *S. neurona* or other *S. neurona*-like organisms in the cat was confirmed through immunofluorescent antibody testing (IFAT) and immunoblot. *Sarcocystis neurona*-like (SN-MUCAT2) and *Sarcocystis neurona* (equine-derived) merozoites were used as the antigen. The patient sample was serially diluted with a starting dilution of 1:50 and ending with 1:3200 for IFAT testing as previously described.³ Control samples included 3 experimentally inoculated cats.³ Fluorescein-labeled (FITC), affinity-purified antibodies (KPL) directed against feline-specific IgG were diluted 1:100 in PBS and added in 10 µL aliquots to each well. The endpoint titer was the last serum dilution showing distinct, whole parasite fluorescence. Immunoblot analysis was performed as previously described including control samples from previously inoculated cats.³

The serology indicated a 1:400 titer by IFAT. The immunoblot (Figure 3) showed that the patient's (lanes 1–3 and 7) serum IgM and IgG antibodies reacted with 14 kDa and 28 kDa antigens. The reactivity of the control experimentally infected cat showed strong IgM reactivity at 14 days post-inoculation (DPI) and strong IgG reactivity at 50 DPI for these antigens. The strength of the reactivity for the experimentally infected cat was much stronger than the patient.

Molecular diagnosis of the *Sarcocystis* sp. was performed by analysis of the small subunit ribosomal RNA (ssRNA) gene^{4,5}, a fragment of the rRNA internal transcribed spacer 1 (ITS-1)⁶, *S. neurona* Surface Antigen Gene 2 (SnSAG2)⁷ and mitochondrial cytochrome c oxidase subunit gene (COX1)⁸, using the reported primers for PCR and subsequent DNA sequencing of the amplicons. In addition, primer pair JNB25/JD396 was used as an exclusion marker for *S. felis* as this primer pair is reported not to amplify *S. felis*.^{9,10} Genomic DNA was extracted from 100 µL of EDTA blood using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Primer pair JNB25/JD396 was used to amplify a genomic fragment of unknown function using conditions modified for real time applications as follows: 94°C for 10 min, followed by 35 cycles of 94°C for 30 s, 50°C for 60 s, 60°C for 45 s, with the last cycle containing an extension time of 7 min at 60°C.⁹ The negative controls included DNA extracted from uninfected normal feline blood and sterile water. Positive controls included DNA extracted from culture-derived *S. neurona* (SN-MU1)¹¹, *S. neurona*-like (SN-MUCAT2) merozoites³, and culture-derived *Neospora caninum* (VMDL-1) tachyzoites.¹² Following PCR, the amplification product was visualized on SYBR green stained gels.

All of the PCR reactions produced the expected sized product or melting temperature peak. To confirm the identity of the PCR amplicons, DNA sequencing was performed on the amplicons using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at The Ohio State University Plant-Microbe Genomics Facility. The resulting forward and reverse sequences were aligned using Vector NTI software (Thermo Fisher Scientific, Waltham, MA, USA). The DNA sequences were compared to published sequences using BLAST analysis available through the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The COC primers, developed against a conserved region of coccidian nuclear small-subunit ribosomal RNA gene and frequently used as screening for coccidian parasites¹³, generated an approximately 300- base pair product. The DNA sequencing generated a 295- base pair sequence. For the ssRNA gene, BLAST analysis showed the greatest match to *Sarcocystis*

felis (AY656815) and *Sarcocystis neurona* (AY628219). Over the COC amplified region there was only a single nucleotide difference at nucleotide 37 (G to T) in reference to GenBank sequence AY628219.

The ITS primers, developed against the internal transcribed spacer 1 region of nuclear ribosomal DNA⁶, generated an approximately 200 base pair product. The DNA sequencing generated a 196- base pair sequence. The ITS amplified region from the unknown parasite showed the most similarity to *Sarcocystis neurona* (AY082644) and *S. dasypi* (AY082631) as it was 100% identical over the region compared and was distinguishable with only 46% identity to the reported partial ITS1 sequence for *S. felis* (KC160207). Furthermore, the SnSAG2 amplicon was 100% identical to *S. neurona* (333 base pair compared, GenBank GQ851952), and COX1 demonstrated 100% identity to partial 475 base pair *S. neurona* sequence (GenBank KF854272) available. The primer pair JNB25/JD396 amplified an approximately 330- base pair fragment from the DNA of the unknown parasite.^{9,10}

DISCUSSION

Finding intracellular and extracellular zoites in the peripheral blood smear suggests systemic parasitemia in the cat described here. Serological and molecular based testing of this cat's blood identified a *Sarcocystis* sp., similar to *S. neurona*. *Sarcocystis* are microscopic, unicellular, obligate intracellular parasites of the phylum Apicomplexa. Sarcocystidae (cyst-forming coccidian) and Eimeriidae (oocyst-forming coccidian) are 2 well-studied families that have genera in which domestic and wild felids are important definitive hosts, including genera *Toxoplasma*, *Sarcocystis*, and *Isospora*.^{1,14,15}

Given the clinical presentation of the cat and the high seroprevalence in domestic cats in general, the primary differential diagnosis in this case was a *T. gondii* infection subsequent to acute exposure or reactivation of a chronic infection after immunosuppression. In older cats, the clinical signs of toxoplasmosis are attributable the spread of tachyzoites after initial acute exposure or reactivation of a chronic infection after immunosuppression.¹⁵ Cats co-infected with FIV and *T. gondii* generally present with more severe clinical signs, including pneumonitis and hepatitis.¹⁶

Diagnosis of *T. gondii* infection antemortem is generally achieved by serology.¹⁵ False negatives in IgM can rarely occur, especially in peracute infections, as IgM typically does not significantly increase until 2 weeks post-infection and may never be present in 10–20% of cats with concurrent FIV infection.^{17,18} Even with the possibility of false negative serology results, one would expect positive PCR results from whole blood, given the visual evidence of organisms on peripheral blood smear examination, as *T. gondii* can be detected via nested PCR with as few as 5 organisms per mL of peripheral blood.¹⁹

With the initial diagnosis of toxoplasmosis excluded, identification of an alternative species was pursued. Subtle visual morphologic evaluation of the zoites length and width suggested *Sarcocystis* merozoites rather than tachyzoites of *Neospora* (6µm×2µm) or *Hammondia* (8µm×2µm).^{14,20–22} The immunocytochemistry documented the reaction of the organisms reacted with *S. neurona*-specific monoclonal antibodies. The DNA sequence of the zoites

was most consistent with *S. neurona* or *S. dasypi*. Although DNA sequencing may not allow for reliable differentiation between these species when highly conserved, slowly evolving DNA regions are used, and there are close phylogenetic relationships among these *Sarcocystis* species.⁶ However, one of the advantages of using conserved regions is the availability of published primers, methods, and gene sequences for comparison. In this case, the lack of identity over the ITS1 region when compared to *S. felis* suggests the unknown parasite in the cat's blood was not *S. felis*. The ITS1 sequence is a more rapidly evolving region and can be used to infer phylogeny among closely related taxa.⁶ The resulting amplicon using the JNB25/JD396 primers which does not amplify *S. felis* corroborates that the unknown parasite in the cat's blood is not *S. felis*. Using only slowly evolving conserved genes for species identification within the *Sarcocystis* genus is not completely reliable, as early work demonstrated that *S. falculata* and *S. neurona* thought to be synonymous based on the rRNA gene were later shown to be biologically, antigenically, and molecularly different.^{6,23}

Cats are definitive hosts for at least 11 *Sarcocystis* species and intermediate hosts for 2 *Sarcocystis* species (*S. felis* and *S. neurona*).²⁴ *Sarcocystis felis* is distributed worldwide and diagnosis in wild and domestic felids has been based on microscopic observation of sarcocysts within muscle tissue. Clinical signs associated with *S. felis* infection in cats have not been reported.²⁵ In contrast, other *Sarcocystis* species have been documented to cause clinical disease in cats. A recent case report described *Sarcocystis*-induced encephalomyelitis in a 5-month-old cat. Merozoites were identified in cerebrospinal fluid analysis and sequencing revealed the organism to be either *S. neurona* or *S. dasypi*.²⁶ In that case, the direct agglutination test for *S. neurona* was used, which does not differentiate between IgM and IgG. In the current case, a combination of IFA (IgG) and immunoblot (IgG and IgM) were applied to distinguish acute from recrudescence infection. Low levels of IgM and IgG antibodies were present to the *Sarcocystis* antigens which suggests that this was a recently acquired infection. In contrast, the experimentally infected control cat serum contained detectable IgM parasite-antigen reacting antibodies at 14 days post infection (DPI). At 50 DPI these IgM parasite specific antibodies were no longer detectable as they were replaced with parasite antigen reacting IgG. Also, the antibodies from the cat in this case report did not react as well to the Sn-MUCAT2 antigen compared to an equine-derived *S. neurona* isolate, suggesting variation in antigen cross reactivity which is known to occur among *Sarcocystis* species, including intraspecies.⁷ Finally, the test antigen may not be optimal as *Sarcocystis* species are known to possess cross-reacting antigens and have antigen variation within species.²⁷

This case represents one of several case reports of systemic *Sarcocystis* infections in clinically affected cats.^{26,28,29} Moreover, *Sarcocystis* spp. (including *S. neurona*, *S. neurona*-like, and *S. dasypi*) are found in wild felids.³⁰ As this case was concurrently infected with FIV, the cat was more susceptible to *Sarcocystis* infection. This should highlight that blood from immunosuppressed cats, despite being *T. gondii* negative, could pose a zoonotic risk to those handling the samples. Specifically, a negative serology for *T. gondii* (either IgM or IgG) does not rule out the potential for another protozoal associated clinical infection in felids.

To the authors' knowledge, this is the first report to visualize by light microscopy circulating *Sarcocystis* sp. merozoites in the peripheral blood of a domestic cat. Furthermore, this is the second report of a case in which patient cat presented symptoms considered most likely due to *T. gondii* infection, which on careful cytologic description, molecular characterization, and antibody testing, revealed *Sarcocystis* organisms.²⁶ Therefore, *Sarcocystis* should be considered among differential diagnoses when cats present with suspected systemic protozoal infection.

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Figure 1.

Peripheral blood smear from a sick cat with circulating zoites (arrows). Wright-Giemsa stain, bar 20 μm . Rare extracellular ($\sim 5\text{--}7\mu\text{m} \times 1\text{--}2\mu\text{m}$) organisms with a central round to oval-shaped purple to deep purple vesicular nucleus with coarsely stippled chromatin and light blue cytoplasm are seen throughout the peripheral blood smear (A and B), indicative of *Coccidia* tachyzoites or merozoites. Organisms are rarely seen within lymphocytes (C) and very rarely seen within large mononuclear cells (D).

Figure 2. Immunofluorescent (A) and immunocytochemical staining (B) of peripheral blood smears from a cat with *Sarcocystis neurona* infection, demonstrating intracellular organisms that positively interact with the *Sarcocystis neurona* 2G5-2 monoclonal antibody (arrows), bar 20 μm . Immunofluorescence detected using fluorescein isothiocyanate (FITC) via confocal microscopy, while immunocytochemical detection via 3'3'-diaminobenzidine horseradish peroxidase (DAB-HRP) and hematoxylin counterstain.

Figure 3. Immunoblot reactivity IgM and IgG in sera from a cat infected with *Sarcocystis neurona*, compared to an experimentally inoculated cat with culture-derived merozoites of *S. neurona* like agent, Sn-MUCAT2. The antigen were culture-derived merozoites of Sn-MUCAT2 separated by SDS-PAGE non-reduced proteins. The sick cat is represented in lanes 1, 2, 3, and 7, serially diluted 1:50; 1:100; 1:200 and 1:200; the experimentally inoculated cat pre-immune serum is in lane 4 and 8, diluted 1:200, at 14 days post inoculation (DPI) in lane 5, diluted 1:200, and at 50 DPI, in lanes 6 and 9, diluted 1:200. Approximate molecular sizes are given at the margin in kilodaltons (Kda).

Table 1

CBC and coagulation abnormalities in a sick cat with circulating *Sarcocystis neurona* zoites in peripheral blood at presentation.

Analyte	Units	Patient	Reference Interval
Plasma protein	g/dL	5.6	5.6 – 7.4
Plasma color	-	Moderate icterus	Colorless
HCT	%	37	26 – 47
Platelet count	$\times 10^9/L$	60	150 – 601
MPV	fL	23.0	8.8 – 22.9
Total leukocytes	$\times 10^9/L$	31.4	4.0 – 14.6
Segmented neutrophils	$\times 10^9/L$	25.1	3.0 – 9.2
Band neutrophils	$\times 10^9/L$	3.5	0 – 0.1
Lymphocytes	$\times 10^9/L$	2.8	0.9 – 3.9
Monocytes	$\times 10^9/L$	0	0 – 0.5
Eosinophils	$\times 10^9/L$	0	0 – 0.3
PT	sec	>250	8.7 – 11
APTT	sec	60.3	9 – 13

Table 2

Serum biochemistry abnormalities in a sick cat with circulating *Sarcocystis neurona* zoites in peripheral blood at presentation.

Analyte	Units	Patient	Reference Interval
ALT	IU/L	268	20 – 95
AST	IU/L	414	10 – 35
ALP	IU/L	5	15 – 65
CK	IU/L	1197	70 – 550
Cholesterol	mg/dL	117	65 – 200
Total bilirubin	mg/dL	3.88	0.1 – 0.4
Total protein	g/dL	4.8	5.6 – 7.6
Albumin	g/dL	2.0	2.5 – 3.5
Globulin	g/dL	2.8	3.1 – 4.1
GGT	IU/L	14	2 – 5
Sodium	mmol/L	139	149 – 155
Chloride	mmol/L	111	116 – 123
Glucose	mg/dL	154	64 – 148
Lactate	mmol/L	3.4	0.4 – 3.4